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ORIGINAL PAPER

Phenolic acid and DNA contents of micropropagated *Eryngium planum* L.

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Abstract A protocol for in vitro production of genetically uniform populations of the medicinal plant *Eryngium planum*, rich in selected phenolic acids, has been established. Shoot-tips were collected from axenic seedlings and grown on a Murashige and Skoog basal medium supplemented with 6-Benzyladenine (BA) and Indole-3-acetic acid (IAA). The highest shoot proliferation efficiency (17 shoots per explant) was obtained when 1.0 mg L^{-1} BA and 0.1 mg L^{-1} were added. Proliferating shoots were rooted and transferred to soil (89 % frequency of survival). Flow cytometric analysis of intact (field-grown) and microrpropagated plants revealed that all plants were uniform in genome size and had similar DNA contents. Thin-layer chromatography (TLC) analysis indicated that multiple shoots and roots from in vitro-derived plants produced high amounts of phenolic acids, primarily of rosmarinic acid (RA). Levels of phenolic acids in in vitro-derived plants were similar to those of intact plants. Furthermore, high-performance liquid chromatography revealed that root cultures in liquid medium accumulated substantial levels of RA. Thus, rapid establishment of in vitro-grown organ cultures of *E. planum* can also serve as reliable sources for bioactive compounds.

Keywords Micropropagation · Flat Sea Holly · Phenolic acids · Rosmarinic acid · Root culture · Flow cytometry

Abbreviations

2.4-D	Dichlorophenoxy acetic acid
FW	Fresh weight
IAA	Indole-3-acetic acid
IBA	Indole-3-butyric acid
BA	6-Benzyladenine
CA	Caffeic acid
CGA	Chlorogenic acid
DW	Dry weight
GA_3	Gibberellic acid
HPTLC	High Performance Thin Layer Chromatography
MS	Murashige and Skoog medium
NAA	α-Naphthaleneacetic acid
RA	Rosmarinic acid
RP HPLC	Reverse Phase High Performance Liquid Chromatography
HPTLC	High Performance Thin Layer Chromatography

Introduction

There are approximately 230–250 species of *Eryngium* L. (Sea Holly), and the genus represents the largest group of

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the Saniculoideae subfamily, which is a member of the Apiaceae family. This taxon is widespread in Central Asia, America, Central and Southeast Europe, North Africa and Australia (Wörz 2005; Wörz and Diekmann 2010). There are 26 *Eryngium* L. species described in Flora Europaea (Tutin et al. 1968). Four of these, including *Eryngium planum* L. (Flat Sea Holly), are rare or protected in Poland, and grow only in restricted regions (Żukowski and Jackowiak 1995). *Eryngium* species are of great value for use in traditional European medicines because they contain phenolic acids, flavonoids, triterpenoid saponins, coumarin derivatives, essential oils, and acetylenes. These compounds are associated with diuretic, antidiabetic, expectorant, spasmolytic anti-inflammatory, antinociceptive, haemolytic, and antimycotic properties (Duke et al. 2002; Le Claire et al. 2005; Köpeli et al. 2006; Zhang et al. 2008; Thiem et al. 2010, 2011).

Phenolic compounds, such as rosmarinic acid (RA), an ester of caffeic acid (CA) and 3,4 dihydroxyphenyllactic acid; and chlorogenic acid (CGA), an ester of CA and quinic acid; are known for their astringent, antiviral, antibacterial, anti-inflammatory, and antimutagenic properties (Petersen and Simmonds 2003; Matkowski 2008; Gugliucci and Markowicz-Bastos 2009). They are natural antioxidants, free radicals, and metal scavengers. This makes them, and CA, interesting targets for phytochemical research in the context of human health. This research focuses on the content of these valuable compounds in *E. planum*, and their use as an indicator of the quality of in vitro produced materials.

Plant in vitro cultures are commonly used in the production of phytochemicals because they produce greater yields than intact plants (Amoo et al. 2012; García-Pérez et al. 2012; Szopa et al. 2012). Several in vitro methods are in use for phenolic acid production (mainly for the production of RA). These methods include suspension cultures, organ cultures, and transformed roots (Petersen and Simmonds 2003; Matkowski 2008; Kikowska et al. 2012; Krzyzanowska et al. 2012). However, there are only a few reports on the in vitro culture of *Eryngium* species. Currently only *E. foetidum*, a rare species endemic to the Caribbean, has been successfully micropropagated for medicinal and cosmetic usage (Arockiasamy and Ignacimuthu 1998; Ignacimuthu et al. 1999; Arockiasamy et al. 2002; Martin 2004; Chandrika et al. 2011).

The advantage of using in vitro culture techniques is that propagation of medicinal plants can occur under controlled conditions. However, micropropagated plants may exhibit somaclonal variation caused by polyploidization, aneuploidy, chromosome aberrations, and sequence changes (Ramulu and Dijkhuis 1986; Zhang et al. 1987; Kaeppler et al. 2000). The risk of genetic instability increases with in vitro culture time and subjection to high levels of exogenous plant growth regulators within the culture media (Philips et al. 1994; Endemann et al. 2001). Thus, control of true-to-typeness is important during tissue culture, as well as in the final products. The confirmation of genetic fidelity is of particular importance when producing biological active metabolites. Flow cytometry is a commonly used method for the confirmation of genome size stability (Thiem and Sliwinska 2003; Sliwinska and Thiem 2007; Rewers et al. 2012). We make use of this method to compare the nuclear DNA content of micropropagated plant material with that of initial plants.

The aim of this study was to develop a rapid and reproducible protocol for *E. planum* micropropagation through axillary bud proliferation. This methodology ensures genome size stability and the ability to produce selected phenolic acids in regenerated plants. To our knowledge, the genome size of this species, the in vitro culture protocol, and the capacity of regenerated plantlets to accumulate bioactive compounds have not been previously reported. The presented protocol allows harvesting of high quality raw material from micropropagated plants without a reduction in the medicinal quality and quantity of RA and CGA.

Materials and methods

Plant material

Fruits, basal leaves, and roots of intact *Eryngium planum* L. plants were collected from natural habitats in Poland (Lukaszewo, Kuyavian-Pomeranian province) in August 2008. The voucher specimens were deposited in the Herbarium of the Medicinal Plant Garden at the Institute of Natural Fibres and Medicinal Plants in Poznan (Poland).

To break dormancy, cleaned fruits were first stored in paper bags at 20 °C for 30 days (warm stratification), and then kept in wet sand at 4 °C for 30 days (cold-stratification). Following this pre-treatment, the pericarp and seed coat were mechanically removed using a scalpel. Isolated embryos were washed with distilled water, dipped in 70 % (v/v) ethanol for 30 s, followed by rinsing in 20 % (v/v) commercial bleach (5 % sodium hypochlorite solution), containing 50 µL of Tween 80, for 5 min. Finally, they were rinsed three times in sterilized double-distilled water, and placed on Murashige and Skoog (MS; Murashige and Skoog 1962) basal medium, or on MS medium supplemented with 1.0 mg L^{-1} Gibberellic acid (GA_3; Sigma-Aldrich; St. Louis; MO; USA). Shoot tips of 30-day-old axenic seedlings were the source of explants. Roots of in vitro plantlets were used for root culture induction.

Table 1 Effects of BA and IAA on shoot proliferation from shoot tip explants of *E. planum* on MS medium after 6 weeks of in vitro culture

Growth regulator (mg L^{-1})		Explants that proliferated buds (%)	Shoot number pe explant (±SE)	Shoot length (cm ± SE)	Leaf number per shoot (±SE)
BA	IAA				
0.0	0.0	93.19 ± 1.0^{d}	4.57 ± 0.60^{c}	4.65 ± 0.04^{a}	2.80 ± 0.07^{f}
1.0	0.0	98.36 ± 0.55^{abc}	8.03 ± 0.60^{b}	1.25 ± 0.03^{e}	5.6 ± 0.06^{a}
1.0	1.0	99.79 ± 0.15^{ab}	15.58 ± 0.59^{a}	1.33 ± 0.02^{e}	3.12 ± 0.04^{e}
1.0	0.1	100 ± 0.00^{a}	17.10 ± 0.60^{a}	1.50 ± 0.02^{d}	3.99 ± 0.04^{c}
1.5	0.1	98.19 ± 0.6^{bc}	8.37 ± 0.60^{b}	3.40 ± 0.03^{c}	3.80 ± 0.05^{d}
2.0	0.1	97.85 ± 0.67^{c}	7.63 ± 0.60^{b}	3.80 ± 0.03^{b}	4.53 ± 0.06^{b}

Mean values within a column with the same letter are not significantly different at P = 0.05 using Duncan's Multiple Range test

Culture media and conditions

Culture media consisted of MS basal medium, liquid (root culture) or solidified with 0.8 % (w/v) agar (shoot proliferation and rooting), supplemented with 3 % (w/v) sucrose and plant growth regulators of various concentrations (Table 1–3). All plant growth regulators originated from Sigma–Aldrich (St. Louis; MO; USA). After adjusting pH to 5.8, media were autoclaved at 121 °C for 20 min at 105 kPa. Tissue cultures were incubated in a growth chamber under a 16:8 h photoperiod at 55 μmol m^{-2} s^{-1} light provided by cool-white fluorescent lamps, and a temperature of 21 ± 2 °C. The root cultures were maintained in darkness.

Establishment of shoot cultures

Seedling shoot tips (0.8–1 cm long) were used for induction of shoot cultures. They were placed in 250 cm^3 Erlenmeyer flasks containing 50 cm^3 of MS medium supplemented with 6-Benzyladenine (BA; 0.5–2.0 mg L^{-1}) and Indole-3-acetic acid (IAA; 0.1–1.0 mg L^{-1}) (Table 1). After proliferating shoots, multishoots were divided into single microshoots, or clusters of 3–5 shoots, and transferred to fresh medium every 5–6 weeks. The total number of shoots, and their length, were recorded after 5 weeks of fifth, seventh, or eighth subculture, using at least 10 explants per medium. Half of the shoots were further multiplied via axillary buds on the same media until enough material was available for phytochemical analyses. The other half were rooted and transferred into *ex vitro* conditions.

Rooting of shoots and plant transfer into soil

For rooting, excised shoots were separated and transferred into half or full-strength MS medium, with or without one of the three auxins, IAA, Indole-3-butyric acid (IBA), or α-Naphthaleneacetic acid (NAA), in concentrations ranging from 0.1–0.5 mg L^{-1} (Table 2). They were cultured in 250 cm^3 Erlenmeyer flasks containing 50 cm^3 of culture medium. The rooting percentage, and the number and length of roots, were recorded after 6 weeks of culture. Healthy plantlets with well-developed roots were subsequently placed in plastic pots containing a mixture of sterile soil and sand (1:1 v/v) with 15 % Perlite (Ekodarpol; Dębno; Poland), and covered with glass beakers for 10–14 days. The plantlets that developed in vitro were then transferred into the field. The survival frequency was recorded once plants had hardened and acclimatized. Some roots excised from in vitro-derived plantlets, growing on MS medium containing 0.1 mg L^{-1} IAA, were collected prior to potting for phytochemical analyses.

Table 2 Effects of auxins on rooting of *E. planum* shoots after 6 weeks of in vitro culture

Medium (auxins concentration in mg L^{-1})	Rooting of shoots (%)	Root number per explant (±SE)	Root length (cm ± SE)
½ MS	92.06 ± 1.60^{a}	1.97 ± 0.51^{b}	4.47 ± 0.11^{a}
MS	90.83 ± 1.72^{ab}	2.78 ± 0.47^{ab}	4.23 ± 0.09^{ab}
MS + IAA 0.1	88.24 ± 1.69^{ab}	3.03 ± 0.48^{ab}	3.86 ± 0.09^{c}
MS + IAA 0.5	90.62 ± 1.11^{ab}	3.10 ± 0.53^{ab}	4.05 ± 0.09^{bc}
MS + IBA 0.1	86.09 ± 1.39^{b}	3.33 ± 0.52^{ab}	1.38 ± 0.09^{d}
MS + NAA 0.1	90.58 ± 1.69^{ab}	4.34 ± 0.53^{a}	0.70 ± 0.08^{e}

Mean values within a column with the same letter are not significantly different at P = 0.05 using Duncan's Multiple Range test

Table 3 Effects of auxin on *E. planum* root culture growth on MS medium

Auxin (0.1 mg L^{-1})	FW (g)	DW (g)
Control (no auxin)	18.3 ± 1.42^{c}	4.7 ± 0.21^{b}
IAA	21.8 ± 1.52^{bc}	5.1 ± 0.23^{b}
IBA	25.1 ± 1.45^{b}	5.8 ± 0.16^{a}
NAA	32.5 ± 1.23^{a}	6.2 ± 0.28^{a}

Mean values within a column with the same letter are not significantly different at P = 0.05 using Duncan's Multiple Range test

Establishment of root cultures

Root cultures were obtained from 0.8-cm root tips of axenic plantlets, and transferred into MS liquid medium with or without one of the three auxins: IAA, IBA or NAA (0.1 mg L^{-1}) (Table 3). The cultures were grown in 300 cm^3 flasks containing 50 cm^3 of culture medium, on a rotary shaker at 100 rpm, in darkness, and subcultured to fresh medium every 6 weeks. The root biomass from 5–7 passages of stable root cultures, maintained in MS medium fortified with 0.1 mg L^{-1} IAA, was collected for phytochemical analyses.

Flow cytometry

The leaves of 6-week-old seedlings, and one-year-old in vitro shoot cultures, were used for the estimation of nuclear DNA content. Samples were prepared as described by Sliwinska and Thiem (2007), using Galbraith's buffer (Galbraith et al. 1983) supplemented with 1 % (v/v) PVP-10, propidium iodide (PI; 50 µg cm^{-3}), and ribonuclease A (50 µg cm^{-3}). *Petunia hybrida* 'P × Pc6′ (2.85 pg/2C; Marie and Brown 1993) was used as an internal standard. Analyses were replicated 10 times for each plant material. For each sample, 5,000–8,000 nuclei were analysed directly after preparation using a CyFlow SL (Partec GmbH; Münster; Germany) flow cytometer equipped with a high-grade solid-state laser with green light emission at 532 nm, long-pass filter RG 590 E, DM 560 A, and side (SSC) and forward (FSC) scatters. Histograms were analysed using FloMax (Partec GmbH) software. The Coefficient of variation (CV) of the G_0/G_1 peak of *E. planum* ranged from 3.92 to 6.03 %. Nuclear DNA content was calculated using the linear relationship between the ratio of the 2C peak positions *Eryngium/Petunia* on a histogram of fluorescence intensities.

Measurement of phenolic acid content

Plant material from in vitro cultures, shoots from shoot culture, roots of plantlets, roots from liquid culture, and the basal leaves and roots of intact plants were used for chromatographic analyses. Shoots and roots were obtained from in vitro-derived plantlets cultured on MS with 1.0 mg L^{-1} BA + 0.1 mg L^{-1} IAA, and MS with 0.1 mg L^{-1} IAA respectively. Roots from liquid in vitro cultures, maintained in MS supplemented with 0.1 mg L^{-1} IAA, were also phytochemically evaluated. The extracts were preliminary analysed by high performance thin layer chromatography (HPTLC) using specific spray reagents to check the presence of the main groups of secondary metabolites: phenolic acids (rosmarinic, chlorogenic, and caffeic acids), flavonoids, and complexes of saponins (Waksmundzka-Hajnos et al. 2008). The HPTLC analyses were as described previously by Thiem et al. (2010).

Qualitative and quantitative analysis of phenolic acids in methanolic extracts were performed using high-performance liquid chromatography (HPLC). Exact amounts of fresh biomass were dried (60 °C for 1 h, followed by 120 °C for 24 h) to a constant weight. Then, 0.2 g of dried and powdered shoot and root biomass was extracted three times with 15 mL 50 % (v/v) methanol for 30 min at the boiling point temperature of the extractive mixture under reflux. Cooled and filtered extract was then diluted with the

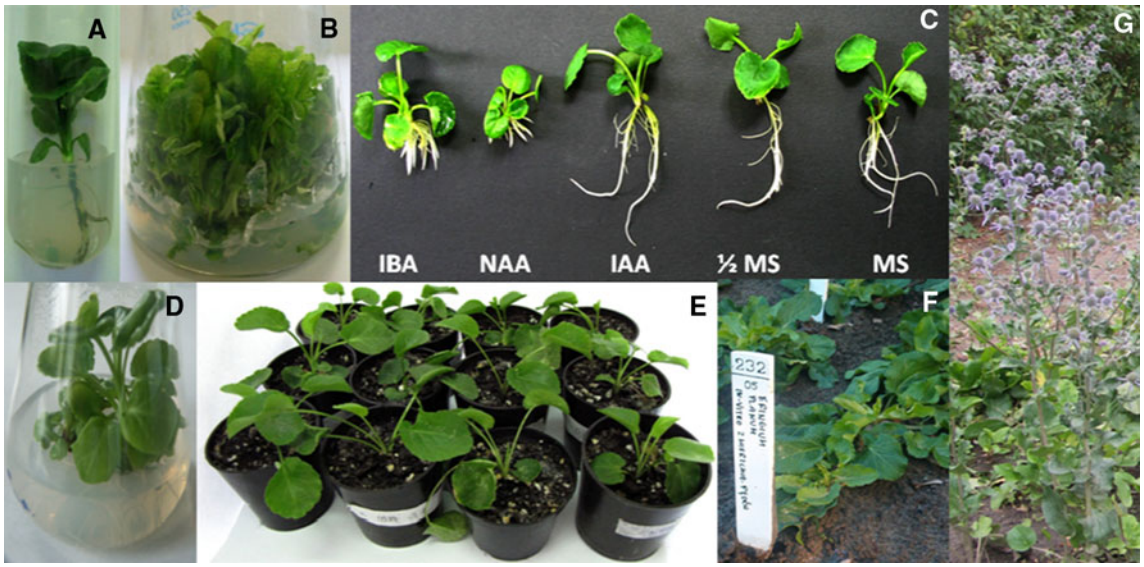

Fig. 1 **a–h** Micropropagation of *Eryngium planum* L. **a** 30-day old seedling. **b** multiplied shoots. **c** roots developed on varied media. **d** single plantlet before acclimation. **e** micropropagated plantlets hardened in glasshouse. **f** plants transferred to the field. **g** flowering plant

methanol 50 % (v/v) to 50 or 100 mL. The solution was filtered through a 0.2-µm filter (Schleicher and Schuell; Dassel; Germany) and 10-µL aliquots were analysed. The phenolic acid content in the methanolic extracts was determined by reverse phase high performance liquid chromatography (RP HPLC), using Merck-Hitachi (Tokyo; Japan) apparatus D-7000 coupled to photodiode array (DAD) on a LiChrospher 100 250 × 4 mm reversed phase column RP 18e, 5 µm (Merck; Darmstadt; Germany). The solvent system was a linear gradient of acetonitrile/H_2O and phosphoric acid pH 2.2: acetonitrile from 15 to 60 % (v/v) for 40 min; 60 % for 15 min; from 60 to 15 % for 1 min; and 15 % for 9 min. The flow rate was 1 mL/min and the effluent monitored by UV detection at 320 nm. RA, CGA and CA were identified by comparison of their retention times (RT) and UV–VIS spectra with those of authentic standards. The retention times (RT) and on-line UV spectra of detected phenolic acids were identified by comparison with standards of RA (RT 13.707 min), CGA (RT 4.600 min) and CA (RT 6.367 min). The calibration was obtained by peak areas of RA, CGA and CA against a concentrated standard solution (mg/100 mL). One concentration was analysed in seven replications. The relative standard deviation of peak areas of RA, CGA and CA were 1.5, 1.3 and 1.1 %, respectively. Chemicals (methanol, acetonitrile, phosphoric acid) were obtained from Merck. Reference substances originated as follows: RA (purity 96.5 %; Sigma–Aldrich; St. Louis, MO, USA), CGA (purity 96.9 %; EDQM, Strasbourg; Japan), CA (purity 99.5 %; Fluka; Buchs; Switzerland). This investigation used 0.001 % methanolic solutions of these standards. Results are means of three separate analyses from three samples of dried plant material.

Statistical analysis

The proliferation of shoots, rooting, and phenolic acid data were subjected to one-way analysis of variance (ANOVA), followed by Duncan's POST-HOC test. ANOVA followed by Student's *t* test was used for flow cytometric results analysis. A two-sided *P* value of 0.05 was used to determine statistical significance. All analyses were conducted using STATISTICA v. 9.1 (StatSoft, Inc. 2010).

Results and discussion

Shoot induction and proliferation

Members of the Apiaceae family produce a schizocarp fruit consisting of two one-seeded indehiscent mericarps, covered with bristles (Heywood 1978). As Apiaceae exhibit endogenous morphological dormancy their propagation by seed is difficult. The seeds germinate readily when fresh, but they become dormant soon after harvest. Therefore, a time-consuming treatment of warm-, followed by cold-stratification is required to break dormancy in stored seeds (Atwater 1980; Njenga 1995). In this study, we developed a rapid in vitro clonal propagation method, using shoot tip explants derived from in vitro-grown seedlings, to increase the number of plants obtained from one seed (Fig. 1a). In a preliminary experiment, the germination of untreated seeds was 6 % on MS medium, and 12 % on MS medium containing 1.0 mg L^{-1} GA_3. Following stratification, and removal of pericarp and testa, germination increased to 60 % on the MS basal medium, and to 89 % on the GA_3 supplemented MS medium. Seedlings were obtained from both types of media.

E. planum has a natural rosette growth habit with plants producing shoot clusters when grown under in vitro culture conditions (Fig. 1b). Because of difficulties involved in separating individual shoots, it is common practice to divide the shoot mass into clusters (George et al. 2008). Under our conditions, the shoot tips of in vitro cultured *E. planum* induced several new buds (9–12), and then proliferated into clusters of shoots. It was easier to transfer clusters of 3–5 axillary shoots to fresh medium than to transfer single shoots, and they grew much better. The addition of exogenous hormones to the MS medium resulted in varied morphogenetic responses; however, multiple shoot buds were formed in all cases (Table 1). After 6 weeks of culture, 98–100 % of explants grown on the hormone-supplemented media formed clusters of 12–15 shoots. Whereas, only 93 % of the explants growing on hormone-free medium proliferated buds. The highest efficiencies of axillary bud proliferation were obtained using either a MS medium supplemented with 1.0 mg L^{-1} BA and 0.1 mg L^{-1} IAA (17 shoots per explant), or a MS medium containing 1.0 mg L^{-1} BA and 1 mg L^{-1} IAA (almost 16 shoots per explant). Shoot proliferation decreased significantly under higher concentrations of BA, or when no IAA was present. Higher concentrations of BA (whether applied alone, or in combination with auxin) are known to decrease the number of new microshoots per explant in other for 12 months Apiaceae such as *Thapsia garganica* (Makunga et al. 2003), *E. foetidum* (Gayatri et al. 2006), *Crithmum maritimum* (Grigoriadou and Maloupa 2008), *Arracacia xanthorrhiza* (Sliva et al. 2010), and *Anethum graveolens* (Jana and Shekhawat 2012). In contrast, the efficiency of shoot proliferation in *Centella asiatica,* and *Vanasushava pedata* was seen to increase in concentrations of up to 5.0 mg L^{-1} BA (Tiwari et al. 2000; Karuppusamy et al. 2006). Karuppusamy et al. (2006) also reported that IAA had a positive effect on shoot length; this was not observed in our study. BA and IAA have been shown to induce organogenesis in *E. foetidum*

Table 4 Nuclear DNA content in leaves of *E. planum* obtained from seedlings and in vitro shoot culture

Plant material	DNA content (pg/2C ± SE)
Leaves from seedlings	1.805 ± 0.0035
Leaves of shoot culture in vitro	1.815 ± 0.0045

Values not significantly different at $P = 0.05$ (t-Student's test)

(Arockiasamy et al. 2002). In our study the shoots of *E. planum* exhibited high vigour, had well-expanded leaves, and unlike *E. foetidum* did not produce exudates (Mahamed-Yasseen 2002). When media were supplemented with a higher concentration of BA, the shoots were observed to be longer than those growing on medium containing 1.0 mg L^{-1} BA, even when the concentration of IAA was not changed. The highest leaf number per developed shoot occurred after culturing on MS medium with BA alone. Sporadic and very limited callus formation occurred at the base of the shoot following culture initiation (1–3 subcultures). Higher concentrations of BA were conducive for callus formation (data not shown). Similar morphogenic responses have been recorded for *E. foetidum* and *C. asiatica* (Ignacimuthu et al. 1999; Tiwari et al. 2000). *In vitro*-derived shoot-tip explants exhibited high regeneration potential with no evidence of decline even after 8–10 subcultures of mass propagation.

Rooting of shoots and plant transfer into soil

The crucial steps of micropropagation are the rooting and acclimation of in vitro plantlets. Following 60 days of culture on proliferation medium, some of the *E. planum* shoots spontaneously formed roots. The remaining shoots were transferred to different rooting media, either with or without supplemental auxin (Table 2). The healthy in vitro-regenerated microshoots formed vigorous roots, and had a high frequency of rhizogenesis (86–92 %). The emergence of adventitious roots occurred after 2 weeks on auxin-containing medium, and 3 weeks on auxin-free medium. On all rooting media tested, microshoots underwent direct root induction, and exogenous auxin was not essential to induce roots. In contrast, in *C. asiatica* and *A. xanthorrhiza*, no in vitro-derived shoots rooted on auxin-free MS media (Tiwari et al. 2000; Sliva et al. 2010). The roots formed by *E. planum* were initially whitish, but subsequently changed to dark brown when the shoots had matured. This might be due to an increased concentration of secondary metabolites. The addition of auxin, especially IBA and NAA, led to a slight increase in the number of roots, and a decrease in their length (Fig. 1c).

The rooted plantlets were successfully hardened to *ex vitro* conditions (Fig. 1d and e), with approximately 89 % of plantlets surviving the transition from tissue culture to the field. In comparison, the survival rate of *A. graveolens* plantlets was 60 %, and that of *A. xanthorrhiza* 62.5 % (Sliva et al. 2010; Jana and Shekhawat 2012). The in vitro-micropropagated plants grew well in the experimental plot, were morphologically uniform, and produced seed in the second season of vegetative growth (Fig. 1f and g). Furthermore, the plants were vigorous, and had well-developed roots. No detectable differences in growth characteristics were observed between in vitro-micropropagated plants and the initial intact plants.

Establishment of root cultures

When grown in phytohormone-supplemented medium, untransformed root cultures usually exhibit efficient growth of biomass, and no genetic modification of the genome occurs. Apiaceae roots have the potential to accumulate secondary metabolites, making them a good source for the production of therapeutic compounds. (Ekiert 2000; Martin et al. 2008; Murthy et al. 2008). Therefore, it is important that successful techniques for the mass culture of Apiaceae

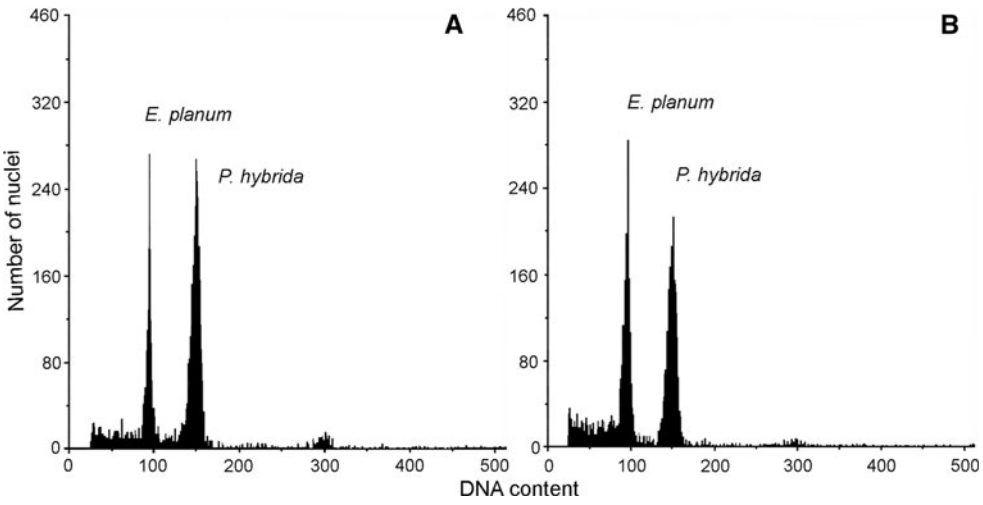

Fig. 2 Histograms of nuclear DNA content obtained after flow cytometric analysis of the PI-stained nuclei isolated simultaneously from the leaves of *Petunia hybryda* (internal standard) and *Eryngium planum*: **a**-seedlings; **b**-regenerated plantlets

species be developed. *E. planum* root growth in liquid MS media was initially very slow, but became rapid after 50 days of culture. As with plantlets, the colour of the roots produced in liquid culture was age dependent; the first roots produced were initially white, but changed in colour, via golden yellow, to brown. These changes in colour may be related to an accumulation of secondary compounds (Martin et al. 2008). The roots showed high viability when liquid MS medium was supplemented with one of the three auxins, IAA, IBA or NAA. The fresh mass of roots grown in media containing auxins was greater than that of roots grown in an auxin-free control; it was greatest in rooting medium supplemented with 0.1 mg L^{-1} NAA (Table 3). The roots cultured in this medium were thicker than those maintained in medium containing IAA or IBA (data not shown). The presence of IBA in a medium led to the production of roots with many laterals. Root growth is known to be controlled by hormones (especially auxins) which affect the genetic and metabolic program of root development.

Nuclear DNA content

Confirmation of genome size stability is of particular importance in plants used as a source of therapeutic compounds because the presence, concentration, and composition of secondary metabolites should remain unchanged after micropropagation. *In vitro* culture often promotes genetic disturbances, and these can result in somaclonal variation; therefore, the control of genome size is desirable. Flow cytometry is widely used to establish DNA content and genome size in micropropagated medicinal plants (Thiem and Sliwinska 2003; Sliwinska and Thiem 2007; Makowczyńska et al. 2008). In this study, the regeneration of *E. planum* L. was performed through axillary bud proliferation without a callus phase. The 2C DNA content of leaves in the control seedlings and in shoots cultured in vitro was similar (approximately 1.81 pg, no statistical difference; Table 4; Fig. 2). Therefore, the nuclear DNA content of in vitro-propagated *E. planum* was maintained during long-term in vitro culture (12 subcultures). Similar genome size stability has been reported in other taxons propagated in vitro, including medical plants such as *Oenothera paradoxa, Inula verbascifolia, Rubus chamaemorus, Solidago virgaurea* and *S. graminifolia (*Winkelmann et al. 1998; Palomino et al. 1999; Sliwinska and Thiem 2007). In contrast, in vitro clones of *Pueraria lobata* have a lower nuclear DNA content than control seedlings (Sliwinska and Thiem 2007). Previously reported estimations of *Eryngium* species genome size are based on Feulgen microdensitometry, and recorded as 1.4 pg/2C for *E. coeruleum*; 2.9 pg/2C for *E. variifolium*; and 5.6 pg/2C for *E. giganteum* (Le Coq et al. 1978). To our knowledge, the genome size of *E. planum* has not previously been estimated.

Phenolic acid accumulation in biomass derived from in vitro cultures

Members of Apiaceae are used for the production of many important bioactive compounds, and these have wide-ranging biological and pharmacological activities (Ekiert 2000). Unfortunately, *E. planum* produces bioactive secondary metabolites in low quantities. This necessitates the development of alternative ways for the production of phenolic acids, especially RA, from *E. planum*. The use of differentiated in vitro organ cultures, especially adventitious root cultures, often results in efficient growth and facilitates the stable production of pharmaceutical compounds. Consequently, this technique has been attempted in many plants that have medicinal value (Murthy et al. 2008). In our study, the accumulation of selected phenolic acids (RA, CGA, and CA) in the shoots and roots of in vitro regenerated plantlets, and in liquid root cultures, was confirmed using HPTLC and HPLC.

Table 5 Content (mg g^{-1} DW) of three phenolic acids detected in methanolic extracts from different in vitro cultures and intact plants of *E. planum*

Plant material	Content of phenolic acids			
	RA (±SE)	CGA (±SE)	CA (± SE)	RA + CGA + CA
Intact plants				
Basal leaves	0.081 ± 0.007^{c}	0.116 ± 0.006^{a}	0.003 ± 0.000^{cd}	0.200 ± 0.013^{c}
Roots	0.055 ± 0.000^{c}	0.019 ± 0.000^{c}	0.002 ± 0.000^{d}	0.076 ± 0.000^{d}
In vitro cultures				
Shoot culture (basal leaves)	0.261 ± 0.003^{b}	0.066 ± 0.003^{b}	0.007 ± 0.000^{b}	0.334 ± 0.006^{b}
Roots of regenerated plantlets	0.056 ± 0.000^{c}	0.013 ± 0.000^{c}	0.005 ± 0.000^{bc}	0.074 ± 0.001^{d}
Root culture	0.595 ± 0.035^{a}	0.059 ± 0005^{b}	0.024 ± 0.001^{a}	0.678 ± 0.030^{a}

Mean values within a column with the same letter are not significantly different at P = 0.05 using Duncan's Multiple Range test

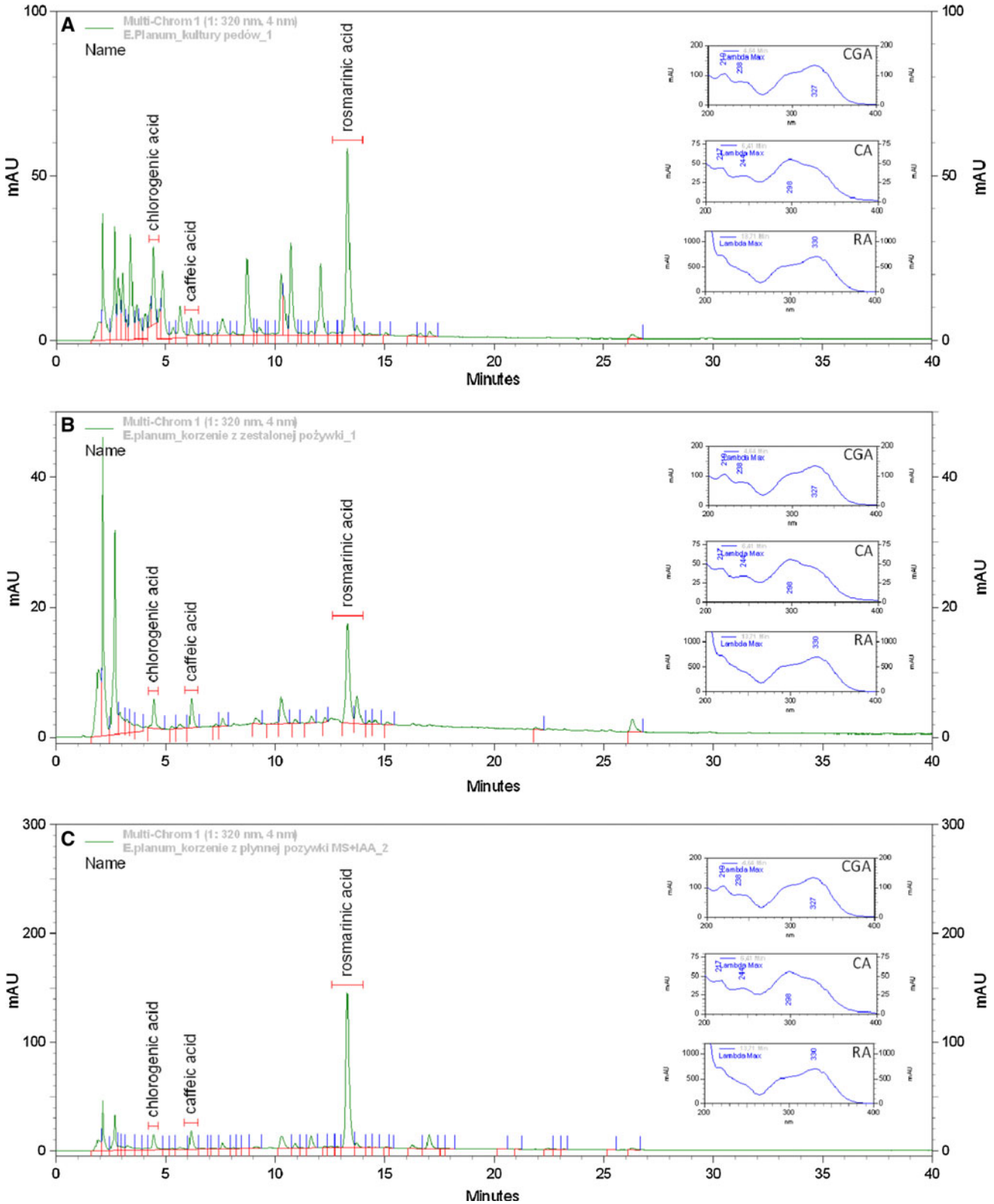

Fig. 3 HPLC chromatograms of the extracts and UV spectra of the investigated phenolic acids in **A** shoots culture in vitro **B** roots from in vitro plantlets and **C** root culture in vitro

HPTLC analyses of ethanolic extracts from regenerated plantlets revealed that in vitro-derived plants and shoots produced phenolic acids, flavonoid compounds, and several saponins. Furthermore, phenolic acids and saponins were present in plantlet root extracts and liquid root cultures. Qualitative and quantitative HPLC analyses of the methanolic extracts from the in vitro cultures confirmed the presence of RA, CGA, and CA in all of the examined materials (Table 5; Fig. 3). Our previous phytochemical analyses had indicated that undifferentiated cultures of *E. planum* cell suspension and callus are able to produce selected phenolic acids (Kikowska et al. 2012). Unfortunately, the accumulation of many bioactive secondary metabolites in the cultured cells was too low to be of use in phytochemical production. This was despite our extensive efforts to optimize cell line growth and production of bioactive compounds. This is thought to be a result of metabolism being controlled in a tissue-specific manner. Cultured undifferentiated cells often partially or totally lose their biosynthetic ability to accumulate secondary products (Murthy et al. 2008). Consequently, we focused our efforts on selected phenolic acid accumulation in in vitro plant-derived organ cultures. The main phenolic compound found in most tested extracts was RA, followed by CGA, and CA. At its highest, the RA content in root cultures was ten and sevenfold higher than in the roots and basal leaves of intact plants, respectively. The use of in vitro conditions also resulted in increased RA production in the leaves of shoot cultures, in comparison with the basal leaves of intact plants. The aerial organs of plants accumulated more CGA than the roots. CA was produced in very low concentrations in all plant samples. Generally, the aerial parts of intact plants and regenerated in vitro cultures had a higher phenolic acid content than the under-ground parts. In intact plants, basal leaves accumulated twofold more phenolic acids than the roots. Similarly, the basal leaves of shoot cultures had a 4.5-fold greater phenolic acid content than the in vitro-regenerated plantlet roots. Interestingly, root cultures grown in liquid medium accumulated an almost tenfold greater amount of phenolic acids than the roots derived from in vitro-regenerated plantlets or intact plants. We observed that there was a difference in the sum of the three analysed phenolic acids in plant organs (basal leaves, or roots) when using plant material sourced from different environments (natural habitat, or in vitro cultures). The differences in analysed phenolic acid content of the organs may be a consequence of the complexities of secondary metabolite production. These processes involve biosynthesis, transport, storage, and degradation steps. The completion of secondary metabolite production depends on equilibrium between these steps, and therefore can vary between different organs and cultures (Wink 1989).

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